Reference: *Biol. Bull.* **182**: 409–415. (June, 1992)

Proline Synthesis During Osmotic Stress in Megalopa Stage Larvae of the Blue Crab, *Callinectes sapidus*

RONALD S. BURTON

Program in Evolutionary Biology, Department of Biology, University of Houston, Houston, Texas 77204-5513

Abstract. The free amino acid (FAA) pool of individual *Callinectes sapidus* megalopas acclimated to 100% seawater averaged over 56% larger than that of 50% seawater acclimated megalopas. Most of the difference was due to a four-fold increase in proline concentration at the higher salinity. In 100% seawater, proline comprises 64% of the total FAA pool in megalopas; this contrasts with the role of proline in adult tissues where it never exceeds 25% of the total FAA pool. Metabolic tracer studies using ^{14}C-glucose and ^{14}C-glutamate as radiolabelled precursors showed that *de novo* synthesis of proline was very low unless induced by hyperosmotic stress. The induction of the synthetic pathway was inhibited by cycloheximide, a protein synthesis inhibitor. These results suggest that the induction of proline synthesis is regulated by the synthesis of either one of the enzymes catalyzing the three steps in the glutamate to proline pathway or a protein acting to stimulate the activity of one of those enzymes.

Introduction

The adjustment of intracellular free amino acid (FAA) concentrations plays an important role in acclimation to salinity change in Crustacea and a diversity of other marine invertebrate taxa (see reviews by Florkin and Schoffeniels, 1969; Gilles, 1975, 1979; Schoffeniels, 1976). High intracellular FAA concentrations apparently function to balance high inorganic ion concentrations in the hemolymph of animals exposed to elevated salinity. Only a few non-essential amino acids such as alanine, proline, and glycine are major contributors to the response and show rapid, quantitatively important, changes in concentration following changes in environmental salinity. One or more of these FAA typically obtain intracellular concentrations in excess of 0.1 *M* in seawater-acclimated Crustacea where the total FAA pool may account for as much as 50% of the total intracellular osmolyte pool (Bowlus and Somero, 1979).

While changes in FAA pool sizes have been widely documented, relatively little is known about the regulation of FAA concentrations during osmotic stress. The most widely cited hypothesis involves the direct action of inorganic ions on a key enzyme, glutamate dehydrogenase (GDH, EC 1.4.1.2), which catalyzes the reductive amination of α-ketoglutarate to form glutamate (see Gilles, 1979; Gilles and Pequeux, 1983; Hochachka and Somero, 1984). Increasing medium salinity is postulated to result in increases in intracellular NaCl concentrations that may directly stimulate GDH activity, resulting in the synthesis of glutamate. Because glutamate is the amino group donor for synthesis of alanine and aspartate (and probably glycine) and a direct precursor for proline, the increased glutamate synthesis could drive, by mass action, the synthesis of these other FAAs. Other key enzymes in FAA synthesis are unaffected by changes in inorganic ions (*e.g.*, transaminases), while some involved in FAA catabolism are inhibited by increased inorganic ion concentrations (*e.g.*, serine hydrolyase). Combined, these effects are thought to alter the synthesis/catabolism balance for FAA and result in their accumulation.

Unfortunately, while data continue to support the occurrence of *de novo* synthesis of FAA in response to hyperosmotic stress (*e.g.*, Burton, 1986), few data directly support the above model for regulation of FAA synthesis. The effect of NaCl and other inorganic ions on glutamate dehydrogenase activity *in vitro* has proven to be complex at best, and some investigators now propose that the major change leading to FAA accumulation is not increased synthesis, but rather reduced FAA catabolism (Gilles, 1979; Gilles and Pequeux, 1983). However, Burton (1986,

Received 19 November 1991; accepted 10 March 1992.

1991a, b) has shown that in the euryhaline intertidal copepod *Tigriopus californicus*, detectable proline synthesis is observed only during hyperosmotic stress. After proline accumulates for approximately 24 h, proline synthesis is effectively turned off. This is in contrast to other FAAs (such as alanine, glutamate, and aspartate), which are synthesized continuously under a variety of salinity regimes. Clearly, proline synthesis is not simply driven by mass action following increased glutamate production because glutamate production occurs under all salinity conditions and glutamate pool size does not change markedly during hyperosmotic stress. Similarly, regulation of the proline pool cannot be the result of changes in proline catabolism alone, because such a model could not account for the fact that ^{14}C-labelling of glutamate (from labelled bicarbonate) occurs under constant salinity (50% or 100% SW), while no labelling of proline is observed under these conditions (Burton, 1986). These data are in direct conflict with the mass action synthesis model discussed above.

Recently, by using *in vivo* translation inhibitor studies, we have shown that the induction of proline synthesis in *T. californicus* in response to hyperosmotic stress requires protein synthesis (Burton, 1991b). By providing ^{14}C-(U)-L-glutamate as a proline precursor, evidence was obtained that the ultimate site of action for protein synthesis inhibitors was in the three-step pathway between glutamate and proline. This work suggests that hyperosmotic stress induces the synthesis of one or more of the enzymes in the glutamate to proline biosynthetic pathway or a protein that stimulates the activity of these enzymes. Given that this mechanism for the regulation of FAA metabolism has not been previously documented among marine Crustacea, it was of interest to determine the generality of our *T. californicus* work by performing similar studies on other, taxonomically distant, crustacean species.

Several criteria were important in choosing an appropriate study system for testing the mechanism of induction of proline synthesis. First, the test organism should be a euryhaline osmoconformer where adjustment of FAA concentrations function in salinity acclimation. Second, because proline is only a minor constituent of the FAA pool in some species, a species was needed in which proline was known to be an important contributor to the FAA pool. Finally, for analytic convenience, we sought a small organism because smaller quantities of tracer isotopes are necessary for metabolic studies. One system meeting these criteria is the blue crab, *Callinectes sapidus*, an abundant portunid that experiences substantial salinity variation in its natural estuarine habitat along the Texas coast. The participation of FAA in osmotic acclimation of adult *C. sapidus* has previously been studied (Gerard and Gilles, 1972; Engel, 1977), and proline was found to be a major contributor to the osmolyte pool in each tissue studied. While adult *C. sapidus* is too large for the *in vivo* radiotracer studies needed to address mechanisms of proline synthesis, *C. sapidus* megalopas (dry weight of approximately 0.4 mg) are locally abundant and easily maintained in the lab. In the work described below, osmotically induced changes in FAA concentrations are documented in *C. sapidus* megalopas and the role of protein synthesis in regulating these changes is assessed via *in vivo* application of the protein synthesis inhibitor cycloheximide.

Materials and Methods

C. sapidus megalopas were collected with a hand-pulled beam trawl in shallow water (<1.5 meter) along the sandy Gulf coast beach of Galveston Island, Texas, in early June to August 1991. Ambient salinities ranged from 17 to 37 ppt. Animals were maintained at room temperature (23°C) and acclimated for 3–5 days at 17 ppt (50% seawater = 50% SW) and 34 ppt (100% SW) before being exposed to experimental treatments. Animals were fed commercial flake fish food (Tetramin) during acclimation. *C. sapidus* megalopas were initially identified by comparing them to the description presented in Costlow and Bookout (1959). Numerous megalopas molted to the first crab stage in our aquaria within a few days of capture; these were identified as *C. sapidus* as described in Williams (1984).

Procedures for studying the incorporation of labelled precursors into the FAA pool in individuals of *C. sapidus* were as follows: prior to exposure to precursor-laced medium, animals were pretreated for 1 h with an antibiotic mixture ("AM 4" of Provasoli *et al.*, 1959) in filtered (0.2 μ), buffered (30 m*M* HEPES) commercial (Instant Ocean) artificial seawater (SW) of appropriate salinity. The effectiveness of this antibiotic mixture in preventing contaminating bacterial growth was previously tested (Burton, 1991a). Radioactive precursor, ^{14}C-(U)-L-glutamate, (Sigma Chemical Company, 229.4 mCi/mmol) or ^{14}C-(U)-D-glucose, (Sigma Chemical Company, 255 mCi/mmol) was added to a small volume (5 μCi/150 μl) of medium of appropriate salinity; all media contained the antibiotic mixture. Experimental treatments involving the translation inhibitor cycloheximide also used a 1-h pretreatment period (with antibiotics and cycloheximide) prior to salinity transfer. Transfers between pretreatment and treatment media were carried out by pipetting individual megalopas onto filter paper and then moving them (with a fine forceps) into 1.5 ml microcentrifuge tubes containing the desired treatment medium. Up to six megalopas were treated together in a single tube. Handling was identical for controls and treatments and did not directly result in any mortality. Osmotic concentrations of artificial SW solutions were routinely determined with a hand refractometer and checked with a vapor pressure osmometer (Wescor Model 5500).

Following experimental exposures (typically 3–6 h), animals were individually sacrificed and FAA extracted in 100 μl of 80% ethanol and then dried under vacuum. Samples were resuspended in 60 μl of 0.1 *M* sodium bicarbonate and then reacted with 40 μl of dansyl chloride in acetone (0.5 mg/ml) for 90 min at room temperature to fluorescently label primary and secondary amino groups. FAA analysis was carried out on dansyl derivatives of the FAA using reverse-phase high pressure liquid chromatography (HPLC) (C18 "Hypersil" 5 μ 4.6 × 250 mm cartridge column, Alltech Assoc.) with fluorescence detection; peaks representing FAA were quantified with a computing integrator and were individually collected directly into minivials for liquid scintillation counting (see Burton, 1986, for further HPLC details). Although glycine, taurine, alanine, and proline derivatives were completely resolved, there was some difficulty in resolving dansyl-glutamate from dansyl-aspartate; data for the combined glutamate/aspartate peak are presented here as "glutamate." To determine whether the confounding of glutamate and aspartate would have a significant effect on estimates of glutamate specific activity, one sample from each experimental treatment was analyzed by one-dimensional thin-layer chromatography (TLC), as follows. Three samples and one lane of dansyl-FAA standards (Sigma Chemical) were spotted in four lanes on a 5 × 20 cm polyamide 6 TLC plate (Baker Chemical). Chromatograms were run in a chloroform-t-amyl alcohol-acetic acid (70:30:3) solvent system until the solvent front migrated 15 cm. Glutamate and aspartate pool sizes were qualitatively assessed under UV illumination, and distribution of radiolabel was determined by a 48–72-h autoradiographic exposure. In most cases, the aspartate spot was too faint to be detected by eye. Subsequently, chromatographic regions in the sample lanes corresponding to aspartate and glutamate standards were cut out and eluted for scintillation counting. In all tests, the bulk of the label (minimum 75%) was recovered in the glutamate region. Because none of our qualitative results are significantly affected by reducing the counts recovered in glutamate by such a factor, we concluded that pooling the glutamate and aspartate peaks via HPLC did not introduce significant error into the results presented here.

Levels of FAA measured by HPLC are presented here in units of nanomoles/larva. The mean (±S.E.) wet weight of a larva was 1.37 ± 0.04 mg; dry weight was 0.39 ± 0.02 mg. Although the reported values can, therefore, be converted to more common units (*e.g.*, mmoles/kg tissue water, or mmoles/g dry weight), the fact that whole megalopas were homogenized would make it difficult to compare the values presented here to values reported for adult tissues. This is because our wet weights include gut water content, and our dry weights consist primarily of exoskeleton rather than actual FAA-containing tissue.

Results

FAA pool of Callinectes sapidus *megalopas acclimated to 50% and 100% SW*

Following collection from ambient 100% SW, groups of megalopas were acclimated to 100% and 50% SW for five days as described above. Results of FAA analyses are shown in Figure 1. In 50% SW, taurine and glycine are the dominant FAAs, comprising approximately 42% and 25% of the measured pool, respectively. The total FAA pool of 100% SW acclimated animals averaged over 56% larger than that of 50% SW acclimated animals (one-tailed *t*-test, $P = 0.022$). The only amino acid that contributed significantly to the increased pool was proline (taurine and glutamate actually showed relatively minor but statistically significant decreases); in 100% SW, proline comprised over 64% of the FAA pool.

Incorporation of radiolabelled glucose into the FAA pool

Megalopas were presented with ^{14}C-(U)-D-glucose (5 μCi/150 μl of medium) under three salinity treatments: constant 50% SW (involved transfer between media of the same salinity), constant 100% SW, and immediately following hyperosmotic transfer from 50% to 100% SW. Larvae were sampled at two time points: 3 h and 6 h after treatment. After 3 h of hyperosmotic stress, concentrations of the five FAAs measured had not increased significantly above the 50% SW control (taurine showed a small but statistically significant drop). Although FAA concentrations had not yet changed, analysis of radiotracer incorporation shows evidence of significant changes in FAA metabolism. Although 80–90% of all recovered radioactivity in FAA is in the alanine pool under each salinity treatment, only proline showed significant variation in specific activity among treatments (Fig. 2). Under either constant salinity treatment, proline specific activity av-

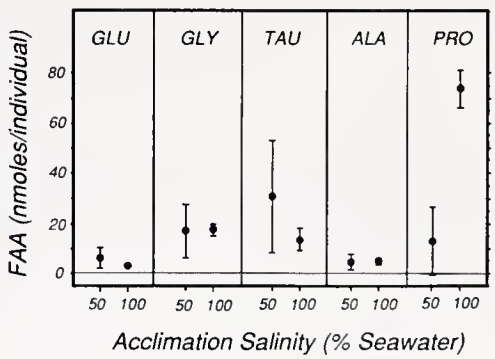

Figure 1. FAA concentrations in individual *Callinectes sapidus* megalopas acclimated to 50% and 100% SW for five days following collection from ambient 100% SW. Error bars are 95% confidence intervals (n = 5 and 6 to the two treatments, respectively).

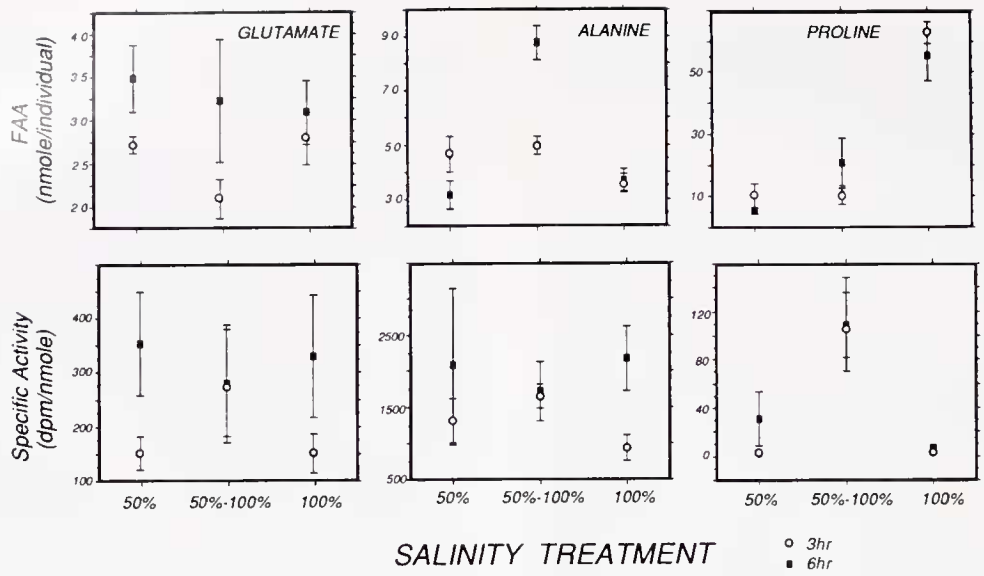

Figure 2. FAA concentrations and specific activities in individual *Callinectes sapidus* megalopas subjected to three treatments: acclimated to 50% and transferred to 50% SW or 100% SW, or acclimated to 100% SW and transferred to 100% SW. ^{14}C-(U)-D-glucose was added and animals were sampled at 3 and 6 h. Four to six individuals were analyzed per treatment. Error bars are ±1 S.E.M. Note scale differences among panels.

eraged less than 2 dpm/nmole while that of glutamate, a direct proline precursor, averaged 95 dpm/nmole, nearly two orders of magnitude higher (note that because aspartate was lumped with glutamate and all counts appear to be in glutamate, this specific activity is an underestimate of true glutamate activity); this fact indicates that proline is essentially not being synthesized *de novo* from glucose carbon under constant salinity conditions. In contrast, proline specific activity in the hyperosmotic stress treatment increased dramatically (to approximately 50% of the glutamate specific activity in the same treatment), indicating the induction of proline biosynthesis by the hyperosmotic stress treatment. Hence, although glutamate and alanine specific activities were not significantly influenced by the salinity treatments, proline specific activity increased by two orders of magnitude within 3 h of hyperosmotic stress. Glycine and taurine showed no label incorporation in any treatment and are omitted from Figure 2.

By 6 h, a significant concentration increase was detected for alanine in the stress treatment over 50% SW controls; while mean proline concentration increased by a factor of four, inter-individual variance was large and the mean difference was not statistically significant (Fig. 2). Again only the specific activity of proline was elevated over constant salinity controls.

Incorporation of radiolabelled glutamate into the FAA pool

Megalopas were presented with ^{14}C-(U)-L-glutamate (5 μCi/150 μl of medium) under both constant salinity (100% SW) and hyperosmotic stress conditions (50–100% SW transfer) to further ascertain that induction of proline synthesis involves the regulation of the glutamate to proline pathway. The high salinity control was employed because if proline is being synthesized under constant salinity conditions, it should be most evident in high salinity where proline pool sizes are large. Results are presented in Figure 3. Although the size of the glutamate pool was slightly larger in the control *versus* the osmotic stress conditions (two-tailed *t*-test, $P < 0.01$), radiolabel recovered in glutamate and glutamate specific activity did not differ. This indicates that a comparable pool of labelled glutamate was available for proline synthesis under both sets of conditions (if anything, slightly more glutamate was available under the control conditions). In contrast, even though the proline pool is significantly larger in control animals ($P < 0.02$), label recovered in the pool and proline specific activity is lower under control conditions ($P < 0.001$ for each measure). Hence, while some label is observed in proline under constant salinity conditions, the near-zero specific activity suggests that the flux from glutamate to proline under these conditions is very low. In fact, if glutamate is the primary precursor of the proline pool, a flux from glutamate to proline should lead to equilibration of the specific activities of the two pools. Paired *t*-tests show that glutamate and proline have different specific activities (within each individual) under control conditions ($P < 0.01$), but not following the 4-h hyperosmotic stress treatment ($P > 0.1$).

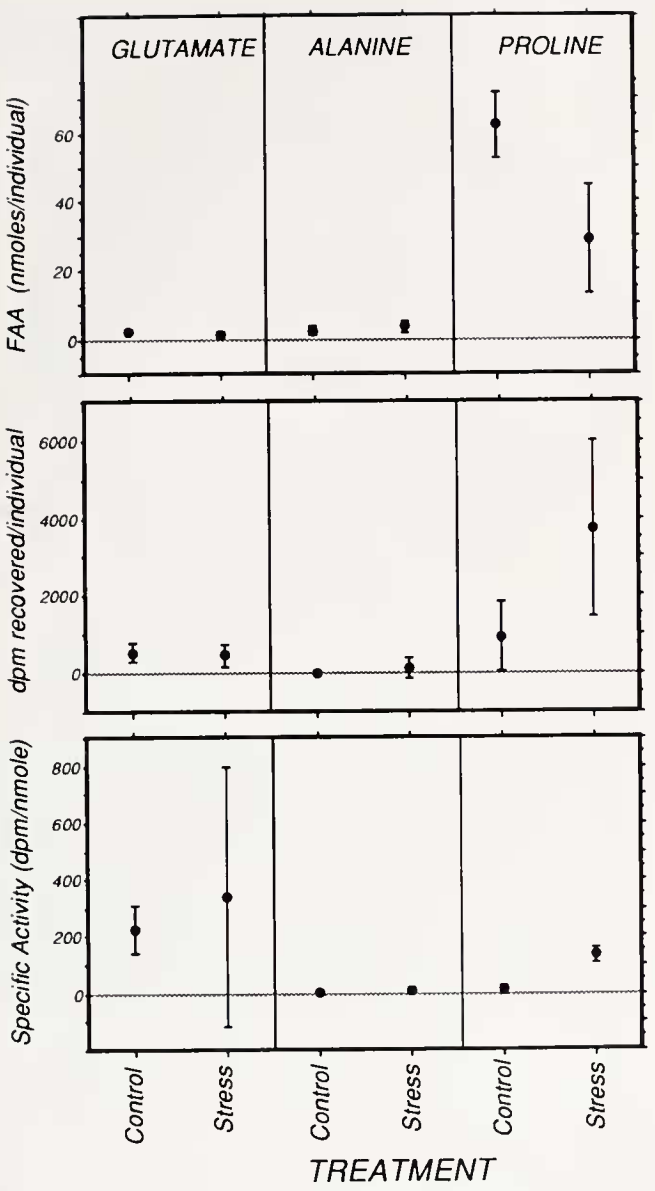

Figure 3. FAA concentrations and specific activities in individual *Callinectes sapidus* megalopas acclimated to 100% SW and transferred to 100% SW (Control) and acclimated to 50% SW and transferred to 100% SW (Stress) for 4 h in the presence of ^{14}C-(U)-L-glutamate. Error bars are 95% confidence intervals (n = 6 individuals per treatment).

Effects of the protein synthesis inhibitor cycloheximide on FAA synthesis

Because the results above clearly demonstrate the induction of proline synthesis from glutamate during response to hyperosmotic stress, we used cycloheximide (CHX) as a protein synthesis inhibitor to address the role of protein synthesis in this induction. Paired groups of megalopas were exposed to hyperosmotic stress, with one group being treated with CHX. Results are shown in Figure 4. Levels of glutamate, labelling of the glutamate pool, and glutamate specific activity do not differ between control and CHX treatments. In contrast, all three measures of proline were affected. Although the size of the proline pool was only slightly decreased by CHX (one-tailed *t*-test, $P < 0.05$), both label recovered in proline and proline specific activity were dramatically reduced by CHX treatment ($P < 0.005$). Interestingly, alanine pool sizes increased by over 40% in the CHX treatment ($P < 0.05$). Neither glycine nor taurine pool sizes were significantly influenced by CHX treatment ($P > 0.25$, data not shown). The magnitude of this change is not enough to compensate for reduced proline synthesis, but it does indicate that

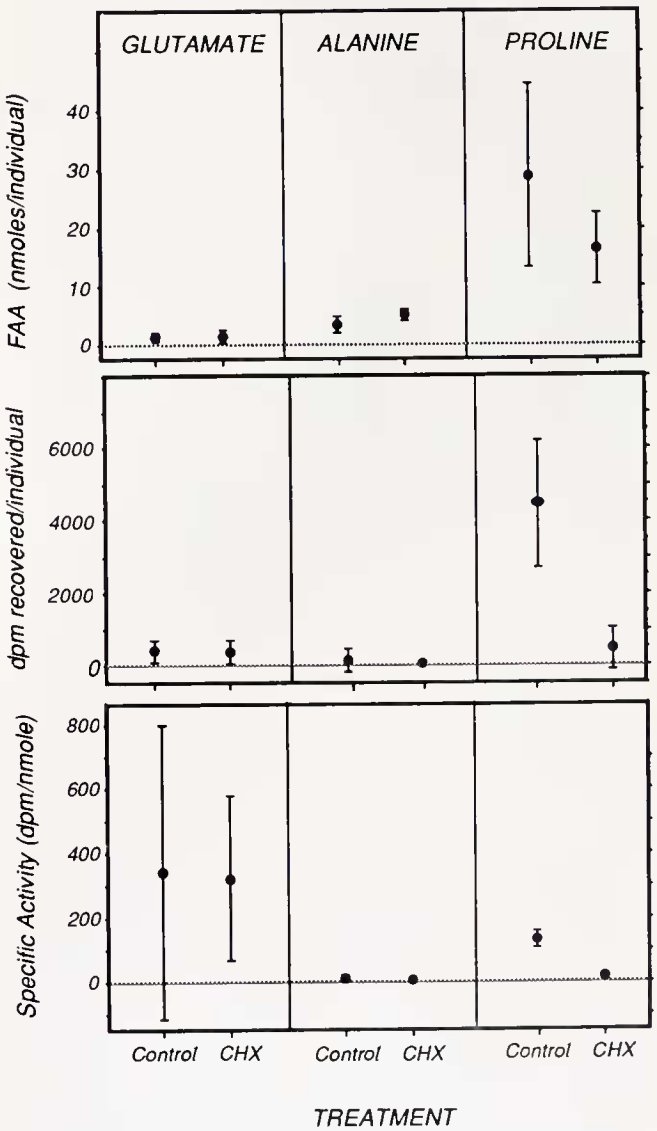

Figure 4. FAA concentrations and specific activities in individual *Callinectes sapidus* megalopas acclimated to 50% SW for 3 days and transferred to 100% SW for 4 h in the presence of ^{14}C-(U)-L-glutamate. CHX groups were pretreated with 10 m*M* cycloheximide for 1 h. Error bars are 95% confidence intervals (n = 6 individuals per treatment).

CHX treatment was specifically inhibitory to proline (but not alanine) synthesis.

Discussion

Although the role of proline accumulation in the hyperosmotic response is variable among the Crustacea, such an accumulation of proline is wide-spread among taxa, having been observed among bacteria (Le Rudulier *et al.*, 1984), fungi (Ho and Miller, 1978), and metaphytes (Boggess *et al.*, 1976), as well as among marine invertebrates (Florkin and Schoffeniels, 1969; Gilles, 1975, 1979; Schoffeniels, 1976). The mechanisms underlying proline accumulation may include protein degradation, uptake from the medium, and *de novo* synthesis. The role of each mechanism varies among taxa: for example, among microorganisms, gram-positive bacteria appear to regulate the synthesis or degradation of proline, whereas gram-negative bacteria achieve accumulation primarily via uptake from the medium (Csonka, 1989).

There is substantial variation among species with regard to the importance of proline in the FAA pool during hyperosmotic response (Claybrook, 1983). Among the Crustacea, proline is insignificant in the FAA pools of some species but the dominant contributor to the FAA pool in others. Furthermore, different tissue types vary dramatically in composition of the FAA pool. While proline is a major contributor to the pool in most adult *C. sapidus* tissues (Gerard and Gilles, 1972), it never accounted for more than 25% of the FAA pool in seawater-acclimated animals. In contrast, our data indicate that proline is the predominant FAA in megalopas, comprising over 50% of the FAA pool. Whether such ontogenetic changes in the composition of the FAA pool are common to other Crustacea has yet to be studied.

The regulation of FAA metabolism in response to osmotic stress among the Crustacea and other marine invertebrates is poorly understood. As discussed above and in Burton (1991a, b), models of direct inorganic ion effects on specific enzymes in FAA metabolism appear to be insufficient to explain the regulation of proline synthesis for two reasons. (1) Patterns of incorporation of radioactive precursors into proline indicate that rate of proline synthesis is nearly undetectable unless induced by hyperosmotic stress (Burton, 1986). (2) Because ^{14}C-labelled glutamate was provided as a precursor and inhibition of protein synthesis prevented proline synthesis which occurred in the absence of inhibitor, we can conclude that protein synthesis inhibition acts somewhere in the glutamate to proline pathway. Based on available information from bacteria and yeast, three gene loci encode the enzymes: γ-glutamyl kinase, γ-glutamyl phosphate reductase, and pyrroline-5-carboxylate reductase (Hayzer and Leisinger, 1980; Tomenchok and Brandriss, 1987). Although work on the proline biosynthetic pathway has not yet progressed to the genetic level among metazoans, available data suggests that homologous gene-enzyme systems are present (Smith *et al.*, 1980; Wakabayashi and Jones, 1983).

The results presented here for *C. sapidus* megalopas are similar to those obtained by Burton (1986, 1991b) for the copepod *Tigriopus californicus* in suggesting that proline synthesis is specifically induced by increases in environmental salinity rather than simply driven by changes in the synthetic rate of a precursor (*i.e.*, glutamate). As in the *T. californicus* system, induction of proline synthesis appears to be dependant on protein synthesis. In both systems, the inhibition of protein synthesis with cycloheximide decreased proline synthesis and accumulation during hyperosmotic stress but significantly increased alanine accumulation. Two possible explanations for the enhanced alanine accumulation follow: (1) By directly preventing the incorporation of alanine and other amino acids into protein, cycloheximide might lead to measurable increases in components of the FAA pool. (2) By preventing the induction of proline synthesis, cycloheximide increases the availability of alanine precursors (*e.g.*, glutamate), thereby stimulating alanine synthesis. In *T. californicus*, cycloheximide treatment resulted in significant incorporation of ^{14}C-label from glutamate into alanine (presumably via glutamate catabolism to malate and then pyruvate, a direct alanine precursor), supporting the latter explanation. This effect was not observed in *C. sapidus*, so the validity of the two hypotheses cannot be resolved with the data available. It should be noted, however, that of the five FAA monitored (glutamate, glycine, taurine, alanine, and proline), only alanine showed increased levels in response to cycloheximide treatment. This suggests that the former hypothesis alone is unlikely to account for the observed pattern of FAA accumulation when protein synthesis is inhibited.

The similarities between proline regulation in *C. sapidus* megalopas and *T. californicus* suggest that the induction of proline synthesis by hyperosmotic stress might be a common regulatory mechanism among the Crustacea. Protein synthesis is clearly required for the induction of proline synthesis in both species. While one must be cautious about generalizing on the basis of only two species, our results suggest a need for molecular tools to determine if the responsible protein is an enzyme in the pathway itself or a regulatory protein of some sort that stimulates existing enzymes to initiate proline synthesis.

Acknowledgments

I thank L. Kordos, J. Bishop, and H. Nguyen for collecting the crab megalopas, and H. Nguyen for technical assistance. J. Bishop and two anonymous reviewers provided thoughtful comments on the manuscript. This work

was supported by Texas Sea Grant, NSF Grant DCB-8811227, and the University of Houston Coastal Center.

Literature Cited

Boggess, S. F., C. R. Stewart, D. Aspinall, and L. G. Paleg. 1976. Effect of water stress on proline synthesis from radioactive precursors. *Plant Physiol.* **58:** 398–401.

Bowlus, R. D., and G. N. Somero. 1979. Solute compatibility with enzyme function and structure: rationales for the selection of osmotic agents and end-products of anaerobic metabolism in marine invertebrates. *J. Exp. Zool.* **208:** 137–152.

Burton, R. S. 1986. Incorporation of ^{14}C-bicarbonate into the free amino acid pool during hyperosmotic stress in an intertidal copepod. *J. Exp. Zool.* **238:** 55–61.

Burton, R. S. 1991a. Regulation of proline synthesis during osmotic stress in the copepod *Tigriopus californicus*. *J. Exp. Zool.* **259:** 166–173.

Burton, R. S. 1991b. Regulation of proline synthesis in osmotic response: effects of protein synthesis inhibitors. *J. Exp. Zool.* **259:** 272–277.

Claybrook, D. L. 1983. Nitrogen metabolism. Pp. 163–213 in *The Biology of Crustacea. Vol. 5,* L. H. Mantel, ed. Academic Press, New York.

Costlow, J. D., Jr., and C. G. Bookout. 1959. The larval development of *Callinectes sapidus* Rathbun reared in the laboratory. *Biol. Bull.* **116:** 373–396.

Csonka, L. N. 1989. Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.* **53:** 121–147.

Engel, D. W. 1977. Comparison of the osmoregulatory capabilities of two portunid crabs, *Callinectes sapidus* and *C. similis*. *Mar. Biol.* **41:** 275–279.

Florkin, M., and E. Schoffeniels. 1969. *Molecular Approaches to Ecology.* Academic Press, New York. 203 pp.

Gerard, J. F., and R. Gilles. 1972. The free amino acid pool in *Callinectes sapidus* (Rathbun) tissues and its role in the osmotic intracellular regulation. *J. Exp. Mar. Biol. Ecol.* **10:** 125–136.

Gilles, R. 1975. Mechanisms of ion and osmoregulation. Pp. 259–347 in *Marine Ecology, Vol. 2, Part 1,* O. Kinne, ed. John Wiley and Sons, London.

Gilles, R. 1979. Intracellular organic osmotic effectors. Pp. 111–154 in *Mechanisms of Osmoregulation in Animals,* R. Gilles, ed. John Wiley and Sons, New York.

Gilles, R., and A. Pequeux. 1983. Interactions of chemical and osmotic regulation with the environment. Pp. 109–177 in *The Biology of Crustacea, Vol. 8,* F. J. Vernberg and W. B. Vernberg, eds. Academic Press, New York.

Hayzer, D. J., and Th. Leisinger. 1980. The gene-enzyme systems of proline biosynthesis in *Escherichia coli*. *J. Gen. Microbiol.* **118:** 287–293.

Ho, K. H., and J. J. Miller. 1978. Free proline content and sensitivity to desiccation and heat during yeast sporulation and spore germination. *Can. J. Microbiol.* **24:** 312–320.

Hochachka, P. W., and G. N. Somero. 1984. *Biochemical Adaptation.* Princeton University Press, Princeton. 537 pp.

Le Rudulier, D., A. R. Strom, A. M. Dandekar, L. T. Smith, and R. C. Valentine. 1984. Molecular biology of osmoregulation. *Science* **224:** 1064–1068.

Provasoli, L., K. Shiraishi, and J. R. Lance. 1959. Nutritional idiosyncrasies of *Artemia* and *Tigriopus* in monoxenic culture. *Annals N. Y. Acad. Sci.* **77:** 250–261.

Schoffeniels, E. 1976. Adaptations with respect to salinity. *Biochem. Soc. Symp.* **41:** 179–204.

Smith, R. J., S. J. Downing, J. M. Phang, R. F. Lodato, and T. T. Aoki. 1980. Pyrroline-5-carboxylate synthase activity in mammalian cells. *Proc. Natl. Acad. Sci. USA* **77:** 5221–5225.

Tomenchok, D. M., and M. J. Brandriss. 1987. Gene-enzyme relationships in the proline biosynthetic pathway of *Saccharomyces cerevisiae*. *J. Bacteriol.* **169:** 5364–5372.

Wakabayashi, Y., and M. E. Jones. 1983. Pyrroline-5-carboxylate synthesis from glutamate by rat intestinal mucosa. *J. Biol. Chem.* **258:** 3865–3872.

Williams, A. B. 1984. *Shrimps, Lobsters, and Crabs of the Atlantic Coast of the Eastern United States, Maine to Florida.* Smithsonian Institution Press, Washington, DC. 550 pp.